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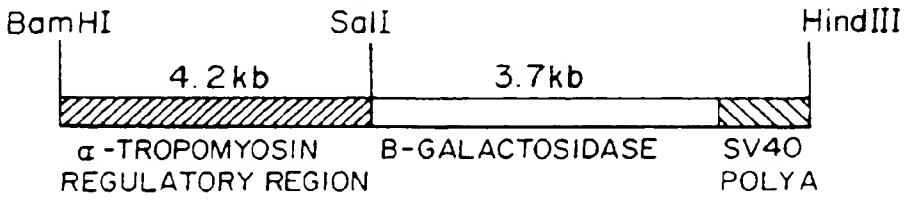

(57) Abstract

A method for testing the ability of a drug to interfere with development of neoplasia is described. A tumor cell having a transformation sensitive reporter unit is introduced into a recipient organism under a condition which reduces the recipient organism's rejection of the tumor cell, a drug is administered to this organism, and a determination is made as to whether the drug has affected the expression of a structural gene that is part of the transformation sensitive reporter unit by assaying for the expressed product of the structural gene. Preferably, the method also includes prescreening the drug by administering the drug to a culture of the tumor cells that have the transformation sensitive reporter unit. Tumor cells and organisms having a transplanted tumor cell are also provided.

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AN IN VITRO/IN VIVO METHOD FOR IDENTIFYING ANTI-NEOPLASTIC DRUGS

This application is a continuation-in-part of U.S. Application Serial No. 07/879,933, filed May 8, 1992, entitled "Anti-Neoplastic In Vivo Drug Screen," by inventors Paul J. Leibowitz, Samuel Wadsworth and Chee-Wai Woon, now pending, and a continuation-in-part of U.S. Application Serial No. 07/999,100, filed December 31, 1992, entitled "Producing Cells for Transplantation To Reduce Host Rejection and Resulting Cells," by inventors Chee-Wai Woon, Samuel C. Wadsworth and Paul J. Leibowitz, now pending.

The entire specifications and drawings of U.S. Application Serial No. 07/879,933, filed May 8, 1992, entitled "Anti-Neoplastic In Vivo Drug Screen," by inventors Paul J. Leibowitz, Samuel Wadsworth and Chee-Wai Woon, and U.S. Application Serial No. 07/999,100, filed December 31, 1992, entitled "Producing Cells for Transplantation To Reduce Host Rejection and Resulting Cells," by inventors Chee-Wai Woon, Samuel C. Wadsworth and Paul J. Leibowitz, are incorporated by reference.

Field of the Invention

This invention relates to a novel in vitro/in vivo screen for identifying anti-neoplastic drugs and to organisms for use in such screens.

Background of the Invention

The identification of anti-neoplastic drugs has long been an objective in medicine. Previous in vivo drug screening assays have targeted drugs that are cytotoxic to cancer

It is such non-cytotoxic drugs that are often the most

desirable because they have fewer detrimental side effects for the organism as a whole.

Moreover, many previous anti-neoplastic drug screening assays have been utilized in vitro test systems. One recently proposed in vitro system uses cells that have a transformation sensitive promoter fused to an Escherichia coli structural gene so that in a transformed cell the structural gene is repressed, and in an untransformed cell the structural gene is expressed. (Kumar, C., Pharm. Tech., 1991, 15: 26-32). It is suggested that this system can be used to identify potential anti-neoplastic drugs. Such a screen, however, suffers from the same limitations that substantially all in vitro screens suffer from, i.e., that no information is obtained about drug availability, drug dosing and timing requirements, tissue specificity of the drug, in vivo drug modification, synergistic effects with other drugs or nutrients ingested by an animal, and effects on the efficacy of the drug relating to an animal's health or age, or the degree of vascularization of a target tumor.

Summary of the Invention

According to the invention, a method for testing the ability of a drug to interfere with development of neoplasia is provided. A tumor cell is introduced into a recipient organism which has an immune system, under a condition which reduces the recipient organism's rejection of the tumor cell. The tumor cell has a transformation sensitive reporter unit which has a structural gene that expresses a product. A drug is administered to the recipient organism which contains the introduced tumor cell. A determination is made as to whether the drug has affected the expression of the structural gene that is part of the transformation sensitive reporter unit by assaying for the product of the structural gene.

The method provides for a transformation sensitive reporter unit which includes a regulatory region that is

operably linked to the structural gene, the regulatory region being either repressed or activated upon transformation of the recipient organism. In preferred embodiments, regulatory regions that are repressed are selected from smooth muscle *a*-actin, skeletal muscle *a*-actin, myosin heavy chain, myosin light chain, *a*-tropomyosin, myo D, myogenin, muscle creatine kinase, collagen 2(*a*)I, H-2K (class I MHC), neu and polyoma; regulatory regions that are activated are selected from glucose transporter GLUTI and multidrug receptor MDR1; and structural genes are selected from Escherichia coli LAC Z, Escherichia coli CAT and Firefly luciferase gene.

Variations of the method of this invention include the tumor cell having the coding sequence for and expressing E19 protein, preferably wherein the coding sequence for the E19 protein is carried on a vector which expresses the E19 protein, or, alternatively, having an inactive MHC gene, so as to alter the presentation of MHC cell surface antigens on the tumor cell and allow introduction of the tumor cell into the recipient organism while reducing rejection by the recipient organism's immune system. Other variations include using an immunodeficient or syngeneic recipient organism, or making the recipient organism immunocompromised by irradiating it or treating it with an immunosuppressant drug such as cyclosporin, azathioprine, FK-506, rapamycin, methotrexate or cyclophosphamide. In certain embodiments, one of the variations regarding the tumor cell is combined with one of the variations regarding the recipient organism.

In the preferred embodiment, the method also includes prescreening the drug by administering the drug to a culture of the tumor cells that have the transformation sensitive reporter unit and determining whether the drug affects

[illegible] organism for use in testing the ability of a drug to

interfere with the development of neoplasia by selecting a tumor cell for transplantation which has a transformation sensitive reporter unit, and transplanting the tumor cell into an organism under a condition which reduces the organism's rejection of the tumor cell.

Yet another aspect of the invention features a method of treating donor tumor cells which have a transformation sensitive reporter unit to reduce recipient rejection caused by MHC class I cell surface antigens. These tumor cells are contacted with E19 protein to alter the presentation of MHC class I cell surface antigens on the tumor cells and allow introduction of the tumor cells into a recipient organism while reducing transplant rejection by the recipient organism's immune system. The tumor cells are provided in a form for introduction into the recipient organism to reduce recipient rejection caused by MHC class I cell surface antigens. In preferred embodiments, the tumor cells are removed from the donor cell culture after the contacting with E19 protein; and the removed tumor cells are introduced into the recipient organism.

In addition, organisms having a transplanted tumor cell with a transformation sensitive reporter unit and, preferably, either the coding sequence for the E19 protein or an inactive MHC gene, are provided. Tumor cells having a transformation sensitive reporter unit and either the coding sequence for the E19 protein or an inactive MHC gene, are also provided.

It is an object of the invention to test the ability of a drug to interfere with the development of neoplasia.

It is another object of the invention to identify anti-neoplastic drugs that are not cytotoxic.

It is another object of the invention to provide an in vivo test for anti-neoplastic drugs that does not rely on changes in tumor size.

It is another object of the invention to provide an in vivo test for putative anti-neoplastic drugs that provides

information regarding drug availability, drug concentration, dosing regimen, tissue specificity, in vivo drug modification and differences relating to age and genetic background of the host organism.

It is another object of the invention to utilize a transformation sensitive reporter unit in an in vivo assay to test for reversal of the transformed state of cells in an organism, in response to a putative anti-neoplastic drug.

It is another object of the invention to utilize an easy, cheap, rapid assay to determine the state of transformation of a cell when subjected to a putative anti-neoplastic drug.

It is another object of the invention to provide a test system in which a cell line can be used for in vitro testing of a putative anti-neoplastic drug and then cells from the cell line transplanted into a host animal for in vivo testing.

It is another object of the invention to provide a combined in vitro/in vivo test system which has the advantages of ease, low cost, rapidity and large numbers of a cell culture system, as well as the advantages of analyzing putative anti-neoplastic drugs in a live animal.

It is another object of the invention to utilize a panel of lineage-specific tumor cell lines to provide a large spectrum of cell and tissue types for assessing the tissue specific efficacy of putative anti-neoplastic drugs.

It is another object of the invention to provide a mammalian model of oncogenesis and tumor growth that is easily assayable.

It is yet a further object of the invention to provide a source of E19 protein in a tumor cell that has a transformation sensitive reporter unit so that upon transplantation of the tumor cell into a recipient organism that is to be used for an in vivo assay for anti-neoplastic

Brief Description of the Drawings

The above and other features, objects and advantages of the present invention will be better understood by a reading of the followingspecification in conjunction with the drawings in which:

Fig. 1 depicts and *a*-tropomyosin promoter-ß-galactosidase fusion construct;

Fig. 2 depicts a collagen 2(*a*)(I) promoter-CAT fusion construct;

Fig. 3 depicts a smoothmuscle *a*-actin promoter-ß-galactosidase fusion construct; and

Fig. 4 depicts pCMVE19NEO:CMV promoter-Adeno-2 E19 fusion plasmid with a neomycin resistance gene.

Detailed Description

The method of this invention provides for testing the ability of a drug to interfere with development of neoplasia, by introducing a tumor cell into a recipient organism having an immune system, under a condition which reduces the recipient organism's rejection of the tumor cell. The tumor cell has a transformation sensitive reporter unit comprising a structural gene which expresses a product. A drug is then administered to the recipient organism containing the tumor cell, and it is determined whether the drug has affected the expression of the structural gene that is part of the transformation sensitive reporter unit, by assaying for the product of the structural gene. In the preferred embodiment, this method also includes the steps of prescreening the drug by administering the drug to a culture of the tumor cells and determining whether the drug has affected the expression of the structural gene from the transformation sensitive reporter unit by assaying for the product of the structural gene.

By neoplasia it is meant a cancerous state. Development of neoplasia means the onset, maintenance or progression of events in a cell which result in a cancerous state.

The term tumor cell is meant to include a cell or group of cells from a tumor or tumor cell line. Tumor cells in culture generally show changes in the properties of normal cells, including anchorage dependence, serum dependence and density-dependent inhibition. Tumor cell lines include (i) characterized human and animal tumor derived lines (American Type Culture Collection, Catalog of Cell Lines and Hybridomas, National Institute of General Medical Sciences' Human Genetic Mutant Cell Repository 1992/1993 Catalog of Cell Lines, DCT Tumor Repository's Catalog of Transplantable Animal and Human Tumors, 1992); (ii) transformed cell lines generated by specific oncogenic insults (for example, introduction of one or more activated oncogenes into a cell line by transfection or viral mediated transduction or exposure to carcinogens); (iii) primary cultures transformed by an oncogenic insult; and (iv) cell lines generated from targeted transgenic expression of specific activated oncogenes or mutant tumor suppressor genes. By transformed it is meant the failure to observe the normal constraints of growth. A transformed cell grows in a much less restricted manner than a normal cell, including generally dividing far more frequently, generally not needing a solid surface to which to attach, generally having reduced serum-dependence, generally piling up into a thick mass of cells instead of being restricted to a thin layer on the surface, and generally inducing tumors when injected into an appropriate organism.

The term organism is meant to include animals. Animals include mammals, birds, reptiles, amphibians and fish. Preferred animals are mammals and preferred mammals are humans, monkeys, pigs, dogs, cats, sheep, goats, cows, horses, rabbits and rodents. Introduction of the tumor cell

applying, grafting, implanting, transplanting and injecting

The tumor cells that are removed from a cell culture for use as donor cells in this invention may be directly introduced into the recipient organism, or they may be stored prior to such introduction.

Introduction of the tumor cell into the recipient organism is performed under a condition which reduces the recipient organism's rejection of the tumor cell. The recipient organism has an immune system. By having an immune system it is meant that prior to imposition of the just mentioned condition, the organism has, or would have but for that condition, an immune system that is normal for that organism. Subsequent to imposition of the condition, the organism may have a normal or abnormal immune system. An abnormal immune system is meant to include one that is partially or completely altered. Altered is meant to include inactive or modified.

Rejection is impairment or destruction of cell structure and/or function to any degree caused by an immune reaction. This invention permits the alleviation of such rejection for a sustained period of time. A sustained time period with regard to the desired result to be achieved is any time period which is long enough to be greater than would otherwise have occurred if the condition of this invention had not been carried out. Such a time period may be short and yet be useful, since the condition imposed with the method of this invention can provide sustained time periods over hours, days, months and years in some cases. Preferably, the reduction in immune rejection is such that the transplanted tumor cells will resist transplant rejection over the life of the organism into which the tumor cells are transplanted, although shorter time periods can be useful for testing the efficacy of putative anti-neoplastic drugs in the recipient organism.

The term reduce transplant rejection is meant to include situations where rejection is eliminated and where rejection is partially eliminated. Transplant rejection can be reduced

with or without the use of supplementary immunosuppressant drugs to enhance the action of the methods and products of this invention. A condition which reduces the recipient organism's rejection of the introduced tumor cell is a condition which is sufficient to effect such a reduction in transplant rejection.

The condition which is imposed includes: (i) the recipient organism's immune system has been altered such that a reduction in rejection of the introduced tumor cell is effected; (ii) the recipient organism's immune system is functioning normally, but the introduced tumor cell has been altered such that it is less susceptible to rejection by the recipient organism's immune system; or (iii) the genetic constitution of the introduced tumor cell is substantially completely identical to the genetic constitution of the recipient organism, i.e., the recipient organism is syngeneic with the introduced tumor cell, resulting in reduced rejection of the introduced tumor cell.

Alteration of the recipient organism's immune system means making the organism immunodeficient or immunocompromised. These conditions result in inadequate functioning of the immune system. Examples of such inadequacy may result from deficient myeloid cells, B-cells, T-cells, stem cells or complement. By immunodeficient it is meant that the immune system is substantially completely non-functional. An example is athymic animals. By immunocompromised it is meant the immune system is partially non-functional. Examples of immunocompromised organisms include organisms that have been irradiated or treated with an immunosuppressant drug. Examples of immunosuppressant drugs include azathioprine, methotrexate, cyclophosphamide, cyclosporin, FK-506 and rapamycin.

the tumor cells so that they have an inactive MHC gene By

contacting it is meant that the donor cell is exposed to E19 protein. Preferably, this contact is intracellular. Most preferably, this intracellular contact is the result of intracellular production of E19 protein.

A preferred method for contacting tumor cells with E19 protein is to construct a tumor cell having the coding sequence for and expressing the E19 protein so as to alter the presentation of MHC (major histocompatibility complex) class I cell surface antigens on the tumor cell to allow introduction of the tumor cell into the recipient organism while reducing transplant rejection by the recipient organism's immune system. The Adenovirus E19 gene encodes a 19 kD glycoprotein from the E3 region of Adenoviruses of the subgroups B, C, D and E. (Paabo et al. PNAS USA, 1986, 83: 9665-9669; Wold et al., J. Biol. Chem., 1985, 260: 2424-2431). By E19 protein it is meant the entire 19 kD protein or a portion thereof. By E19 coding sequence it is meant the coding sequence for the entire 19 kD protein or a portion thereof. The E19 protein has a dominant mode of action. It is believed that E19 protein binds to and prevents MHC class I molecules from exiting the endoplasmic reticulum of the cells. (Paabo et al., Adv. Cancer Res., 1989, 52: 151-163; Burgert et al., PNAS USA, 1987 84: 1356-1360; Cox et al., J. Exp. Med., 1991, 174: 1629-1637). By altering the presentation of MHC class I cell surface antigens on the cell it is meant that MHC class I molecules are at least partially blocked from becoming or acting as cell surface antigens. This alteration in presentation must be sufficient to reduce transplant rejection by a recipient organism's immune system when the tumor cells are introduced into the recipient organism.

A tumor cell can have the coding sequence for the E19 protein by introducing into the tumor cell a vector or other DNA having the E19 coding region. The E19 coding sequence may be operably linked to its own promoter or to another promoter, including a strong general promoter or a tissue or

organ specific promoter. The term vector is meant to include viruses, plasmids, cosmids and YACS. Plasmids include DNA molecules that can replicate autonomously or integrate into a host. DNA from plasmid constructs can be used to introduce the E19 coding sequence into tumor cells grown in vitro by DNA mediated cell transfection methods. The preferred vector is a virus. Preferred viruses include recombinant retroviral vectors, recombinant adenoviral vectors and recombinant Herpes simplex viral vectors. Recombinant retroviral vectors capable of transducing and expressing the E19 coding sequence in tumor cells are produced by transfecting the recombinant retroviral genome into a suitable (helper virus-free) amphotropic packaging cell line. Examples of such cell lines include PA317 and Psi CRIP (Cornetta et al., Human Gene Therapy, 1991, 2: 5-14; Cone & Mulligan, PNAS USA, 1984, 81: 6349-6353). Transfected virus packaging cell lines produce and package the recombinant retroviral vectors, shedding them into the tissue culture media. The retroviral vectors are then harvested and recovered from the culture media by centrifugation as described in Compere et al., MCB, 1989, 9: 6-14. The retroviral vectors are resuspended in 10 mM HEPES.

Confirmation that the E19 coding sequence has been introduced into the tumor cell by one of the above described ways is accomplished by measuring expression of the E19 coding sequence in these cells. Expression of the E19 coding sequence in the tumor cells may be examined by Northern Blot analysis (Sambrook et al., Molecular Cloning: A Laboratory Manual, CSHL, 1989) of total RNA prepared from the cells or by immunoblot analysis with E19-specific antisera (Wold et al., J. Biol. Chem., 1985, 260: 2424-2431).

Once the tumor cells have been contacted with E19 protein

physical structure of the tumor cells during the contacting

step. Such a form requires that the tumor cells be in an appropriate condition for introduction with regard to factors including physiological state, chemical environment and physical condition.

As mentioned above, another possible condition is altering the tumor cell by modifying the genome of the tumor cell so that it has an inactive MHC gene so as to allow introduction of the tumor cell into a recipient organism while reducing transplant rejection by the recipient organism's immune system. MHC genes code for cell surface proteins. Inactivation of an MHC gene for this invention results in altered presentation of MHC antigens in whole or in part on the tumor cell so that the tumor cell will no longer be subject to normal immune surveillance by the recipient organism. The term inactive MHC gene is meant to include inactivation of both alleles of the MHC gene if the inactivation results in a recessive phenotype, or inactivation of one or both alleles of the MHC gene if the inactivation results in a dominant phenotype, or interference with expression of the MHC gene by the presence of an MHC antisense RNA, or interference with presentation of the MHC cell surface antigens on the tumor cell. Having an inactive MHC gene is also meant to include having a deletion of the MHC gene.

Inactivation of an MHC gene is accomplished by standard genetic and molecular methods. Preferably, inactivation is accomplished by gene knockout. An inactivating mutation, for example, a premature stop codon, is introduced as an insertion so as to interrupt the reading frame in an exon of the MHC gene which is carried on a vector. This mutated MHC gene is used to alter the composition of the endogenous MHC gene in the chromosome of the tumor cell by precisely targeted homologous recombination (Dorin et al., Science, 1989, 243: 1357-1360; Thomas and Capecchi, Cell, 1987, 51: 503-512; Cosgrove et al., Cell, 1991, 66: 1051-1066). For example, an invariant subunit component of the functional MHC

molecule, the B_2-microglobulin, is targeted for inactivation (Zijstra et al., Nature, 1989, 342: 435-438).

Antisense RNA is inserted into the cDNA of the MHC gene, either in part or in whole, in the antisense orientation in an expression vector so as to allow the synthesis of a stable antisense message in the cell. Preferably the vector is a mammalian expression vector containing a functional promoter and appropriate polyadenylation sequence. For example, a plasmid construct is introduced into the tumor cell by DNA mediated transfection or a retroviral vector is introduced by infection of the tumor cell. The antisense message so produced anneals with the endogenous MHC mRNA and results in either the double stranded RNA being degraded or in interference with translation (Narayanan et al., Oncogene, 1992, 7: 553-561; Kolch et al., Nature, 1991, 349: 426-428; Murray, Antisense RNA and DNA, Wiley-Liss Inc., 1992).

In certain embodiments of this invention a combination of conditions is employed in order to achieve a more effective reduction in rejection of the introduced tumor cell by the recipient organism. For example, introduction of the E19 coding sequence or an inactive MHC gene into a tumor cell can be coupled with the recipient organism being treated with an immunosuppressant drug or being irradiated.

Regardless of which of the above described conditions is imposed, the tumor cells of this invention have a transformation sensitive reporter unit. The term transformation sensitive reporter unit includes a sequence of DNA that encodes for a product that can be monitored and a sequence of DNA that regulates expression of that product in response to cellular transformation. Expression of the product of the transformation sensitive reporter unit can be either repressed or activated in response to the

embodiments, the transformation sensitive reporter unit is

either substantially completely repressed or substantially completely activated by transformation so that reversal of these states can be easily detected.

The regulatory region of the transformation sensitive reporter unit is a cis-acting DNA sequence which controls transcription of a gene. Sequences within this regulatory region are recognized by regulatory proteins. A regulatory region may be the target of one or many regulatory proteins. A regulatory region includes promoters and enhancers. A promoter is a DNA sequence which contains signals for the start of RNA synthesis. It is a region of DNA that is involved in binding RNA polymerase to initiate transcription. An enhancer is a DNA sequence which alters the efficiency of transcription by increasing the utilization of some eukaryotic promoters. A particular enhancer may be a target for tissue specific or temporal regulation.

Examples of regulatory regions that are repressed upon transformation include the regulatory region from smooth muscle *a*-actin (Leavitt, J. et al., Nature, 1985, 316: 840-842), skeletal muscle *a*-actin (Webster, K.A. et al., Nature, 1988, 332: 553-557), myosin heavy chain (Webster, K.A. et al., Nature, 1988, 332: 553-557), myosin light chain (Kumar, C.C. & Chang, C., Cell Growth & Differentiation, 1992, 3: 1-10), *a*-tropomyosin (Cooper, H.L. et al., MCB, 1985, 5: 972-983), myo D (Lassar, A.B. et al., Cell, 1989, 58: 658-667), myogenin (Lassar, A.B. et al., Cell, 1989, 58: 658-667), muscle creatine kinase (Schneider, M.D. et al., MCB, 1987, 7: 1973-1977), collagen type (I) (Avvedimento, E. et al., 1981, 9: 1123-1131; Schmidt, A. et al., Nature, 1985, 314: 286-289), H-2K (MHC class I) (Vaessen, R.Y.M.J. et al., 1986, EMBO J., 5: 335-341), neu (Yu, D. et al., PNAS USA, 1990, 87: 4499-4503), and polyoma (early and late) (Velcich et al., MCB, 1986, 6: 4019-4025).

Examples of regulatory regions that are activated upon transformation include the regulatory region from glucose transporter (GLUTI) (Kahn & Flier, Diabetes Care, 1990, 13:

548-564), multidrug receptor (MDRI) (Chin et al., Science, 1992, 255: 459-462), and other as yet uncharacterized genes (Garrels & Franza, J. Biol. Chem., 1989, 264: 5299-5312). In addition to a regulatory region, the transformation sensitive reporter unit comprises a structural gene. By structural gene it is meant a sequence of DNA that encodes for a product. The product encoded by the structural gene includes an RNA or polypeptide. The product may be a full length gene product, or it may be a subfragment thereof, or it may be part of a fusion product, provided that it is assayable. In the case of interrupted eukaryotic genes, a structural gene is meant to include sequences which include exons and introns, as well as those that include exons and some introns, or only exons.

A factor to be considered in the choice of a structural gene to be used as the reporter gene is ease of assayability. Examples of structural genes that can be used in this invention are Escherichia coli LAC Z (codes for ß-galactosidase), Escherichia coli CAT (codes for chloramphenicol acetyltransferase), Firefly luciferase gene (codes for luciferase) and transformation sensitive mammalian genes, encoding, for example, *a*-actin, myosin heavy chain, myosin light chain, *a*-tropomyosin, myo D1, myogenin, collagen 2*a*(I), H-2K (MHC class I), neu, phosphoenol pyruvate carboxykinase, and polyoma gene products. Preferred reporter genes include exogenous structural genes so as to avoid any interference in the assays with endogenous background levels of the reporter gene product. The term exogenous structural gene is meant to include a gene that is not normally found in the genome of the tumor cell. The term endogenous structural gene is meant to include a gene that is normally found in the genome of the tumor cell. The most

hybridization. Histochemical methods include the use of

certain compounds as color indicators for enzyme activity of the structural gene. Enzymatic methods include measuring the catalytic activity of the enzyme product of the structural gene. Immunohistochemical methods include using specific antibodies that recognize the polypeptide product of the structural gene. A wide range of antibodies for cellular proteins is available from commercial suppliers such as Sigma Chemical, Boehringer Mannheim and Dako Corp. In situ hybridization includes measuring the levels of mRNA produced by the structural gene with an antisense oligonucleotide probe.

Preferably, ß-galactosidase is assayed histochemically, for example, with X-gal as a color indicator. ß-galactosidase reacts with X-gal to produce a blue color which is easily visualized (Bennerot et al., PNAS USA, 1990, 87: 6331-6335). Assays for chloramphenical acetyltransferase include enzymatic (Gorman et al., MCB, 1982, 2:1044-1051), immunohistochemical (Bullock & Petrusz, Techniques in Immunocytochemistry, Academic Press, 1982), and in situ hybridization assays (Niedobitek & Herbst, Int. Rev. Expt. Pathol., 1991, 32:1-56). Preferably, luciferase is assayed by chemiluminescence (De Wet et al., MCB, 1987, 7: 725-737). The gene products from the transformation sensitive mammalian genes preferably are assayed by antibody reaction.

The transformation sensitive reporter unit preferably is introduced into the tumor cell as an intact unit. For example, an exogenous structural gene may be recombined with a regulatory region in vitro to form a transformation sensitive reporter unit. The regulatory region may be derived from a regulatory region that is normally endogenous to the recipient tumor cell or from a regulatory region that is normally exogenous to the recipient tumor cell. As a result of the in vitro recombination event, the regulatory region becomes operably linked to the structural gene. The term operably linked is meant to include the situation where expression of the structural gene is under the control of the

regulatory region. Such control includes control by either the promoter or enhancer of the regulatory region, or by both.

As an alternative, the transformation sensitive reporter unit may be formed in vivo as a result of recombination between a part of the unit that is introduced into the tumor cell and a part of the unit that is endogenous to the tumor cell. For example, an exogenous structural gene may be introduced into the tumor cell and undergo recombination with the tumor cell's genome (Dorin et al., Science, 1989, 243:1357-1360), resulting in insertion of the exogenous structural gene so that it is regulated by an endogenous regulatory region. Insertion may occur upstream or downstream of the transformation sensitive regulatory region, provided that the regulatory region is operably linked to the structural gene.

Insertion of either an intact transformation sensitive reporter unit, or insertion of part of a transformation sensitive reporter unit should be in a manner so as to result in stable introduction into the tumor cell, i.e., it becomes integrated into a resident chromosome or is maintained in successive generations as an autonomously replicating unit. An example of an autonomously replicating unit is Bovine Papilloma Virus Vector (Elbrecht et al., MCB, 1987, 7:1276-1279). Preferably, the transformation sensitive reporter unit is integrated.

In the preferred embodiment, a potential anti-neoplastic drug is prescreened in tumor cells containing a transformation sensitive reporter unit. A drug which is so identified in this in vitro prescreen is then tested in the in vivo screen of this invention. Preferably, the regulatory region of the transformation sensitive reporter unit is repressed upon neoplastic transformation and the structural

[illegible] resulting in derepression of the structural gene.

Alternatively, the regulatory region is activated as a result of transformation and the structural gene is turned on. In this case, an anti-neoplastic drug will result in repression of the structural gene. In either embodiment, the ability of the drug to reverse expression of the structural gene, as compared to its expression under transforming conditions, identifies the drug as an anti-neoplastic drug.

This invention also includes an organism having a transplanted tumor cell which has a transformation sensitive reporter unit, an organism having a transplanted tumor cell which has a transformation sensitive reporter unit and the coding sequence for the E19 protein, and an organism having a transplanted tumor cell which has a transformation sensitive reporter unit and an inactive MHC gene. This invention further includes a tumor cell for transplantation which has a transformation sensitive reporter unit and the coding sequence for the E19 protein, as well as a tumor cell for transplantation which has a transformation sensitive reporter unit and an inactive MHC gene.

The methods of this invention can be used to make qualitative determinations of the presence or absence of an anti-neoplastic drug in the screening procedures used. In some cases quantitative results as to anti-neoplastic activity can be determined by the level of transcriptional activity of the reporter gene or the level of activity of the reporter gene product.

EXAMPLES

Example 1: Construction of the *a*-tropomyosin promoter-ß-galactosidase fusion plasmid

The promoter region of the rat *a*-tropomyosin gene is fused to the Escherichia coli LAC Z gene, which codes for ß-galactosidase. The regulatory region of the rat *a*-tropomyosin promoter is obtained from plasmid pTM1A. This plasmid contains a 6.5 kb fragment of the 5'-region of

the rat *a*-tropomyosin gene including exons 1, 2 and 3 (Ruiz-Opazo & Nadal-Ginard, J. Biol. Chem., 1987, 262: 4755-4765; Harrera & Ruiz-Opazo, J. Biol. Chem., 1990, 265: 9555-9562). The promoter is isolated as a 4.2 kb BamHI-ApaI fragment which includes the transcription start site. The ApaI end is modified by conventional recombinant DNA techniques to generate a unique SalI site. The ß-galactosidase gene is removed from plasmid pCMV-B (Clontech) as a 3.7 kb XhoI-HindIII fragment. The *a*-tropomyosin-ß-galactosidase fusion construct is assembled by ligating the promoter and reporter gene fragment at the SalI/XhoI sites into the BamHI and HindIII cloning sites of plasmid pUC18 (N.E. Biolabs). The fusion construct is shown in Figure 1.

Example 2: Construction of the collagen 2*a*(I) promoter-CAT fusion plasmid

The promoter region of the human collagen *a*2(I) is fused to the Escherichia coli chloramphenicol acetyl transferase (CAT) gene. The regulatory region is isolated as a 3.5 kb EcoRI/SphI genomic fragment that spans a region 3500 bp upstream of the transcription start site, including 58 bp of the 5'-untranslated region of the human collagen 2*a*(I) gene (Boast et al., J. Biol. Chem., 1990, 265: 13351-13356). The SphI end is modified by linker ligation to generate a HindIII site. The chloramphenicol acetyltransferase reporter gene is removed as a 1.65 kb HindIII-BamHI CAT cassette from a derivative of pSV2CAT (Gorman et al., MCB, 1982, 2: 1044-1051). The fusion construct is assembled by ligating the two gene fragments at the HindIII sites into the EcoRI and BamHI cloning sites of pUC18. The fusion construct is shown in Figure 2.

Example 3: Construction of the smooth muscle *a*-actin promoter-ß-galactosidase fusion plasmid

The promoter region of the human smooth muscle *a*-actin promoter is fused to the Escherichia coli LAC Z gene, which codes for ß-galactosidase. The regulatory region of the smooth muscle *a*-actin gene is isolated as a 960 bp EcoRI-DraIII fragment from the plasmid paAS (Reddy et al., J. Biol. Chem., 1990, 265: 1683-1687). This fragment includes approximately 900 bp of the regulatory region of the promoter and the transcription start site. The ends of the fragment are filled in by conventional recombinant DNA techniques and cloned into the HindIII cloning site of plasmid pCH110 (Clontech) to obtain the fusion *a*-actin promoter-ß-galactosidase construct. The fusion construct is shown in Figure 3.

Example 4: Construction of an Adenovirus E19 expression plasmid

The E3 region of Adeno-2 containing the E19 open reading frame (Wold et al, J. Biol. Chem., 1985, 260: 2424-2431) is subcloned as a 540 bp BamHI fragment in the plasmid pcDNA1NEO (Invitrogen). The resulting construct places the expression of E19 under the transcriptional control of the human CMV (Cytomegalovirus) immediate early promoter (Boshart et al., Cell. 1985, 41: 521-530). The fusion plasmid is shown in Figure 4.

Example 5: Anti-Neoplastic Drug Screen Using Melanoma Cells Having a COL2*a*(I) Promoter-CAT Reporter Unit Transplanted Into a Syngeneic Host

The transformation sensitive reporter unit COL2*a*(I) promoter-CAT is introduced into B16-F1 melanoma cells in culture and the resulting cells are transplanted into a syngeneic host for use in anti-neoplastic drug screening.

A. B16 mouse melanoma cell line

A B16 melanoma cell line (H-2^b haplotype) has been isolated by Fidler (Nature New Biology, 1973, 148-149). Following exposure in culture to lymphocytes from histocompatible C57BL/6 mice immunized against B16, variants of the parental line B16-F1 were selected that are no longer sensitive to lymphocyte-mediated cytotoxicity (Fidler et al., Cancer Res. 1976, 36: 3160-3165). The latter includes B16-$F1^{Lr6}$ and $F10^{Lr6}$, and manifest increased extrapulmonary (liver, lung, bladder) metastasis potential. The parental line B6-F1 produces pulmonary tumor nodules when transplanted (intravenously) into a syngeneic host with low metastatic potential. Neither the lymphocyte-susceptible nor the lymphocyte-resistant B16 cells grow in allogeneic hosts (Fidler & Bucana, Cancer Res. 1977, 37: 3945-3956).

B. B16 mouse melanoma cell culture

B16-F1 melanoma cells are obtained from the DCT Repository (NCI, Frederick, MD) or from ATCC (CRL#6323) (ATCC, Rockville, MD). The cells are maintained in culture as monolayers in plastic flasks (Becton Dickinson Labware, Oxnard, CA) in Eagle's minimum essential media supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), sodium pyruvate 1 mM, nonessential amino acids, L-glutamine 2 mM, MEM vitamin (2 fold), and penicillin-streptomycin (Gibco, Grand Island, NY). All cultures are maintained at 37°C, 5% CO_2 in a humidified chamber. The cells have a doubling time of 16-18 hours. Cultures are fed every 3 days.

C. Transformation sensitive reporter unit construct

A transformation sensitive reporter unit is assembled by

D. Introduction of the transformation sensitive COL2*a*(I) promoter-CAT reporter into B16-F1 melanoma cells in culture

DNA is introduced into B16-F1 cells by the calcium phosphate coprecipitation method (Chen & Okayama, Biotechniques, 1988, 6: 632-638). For selection, the hygromycin resistance gene encoded in the plasmid p220.2 (Sudgen et al., MCB, 1985, 5: 401-413) is introduced along with the COL2*a*(I)-CAT construct at a ratio of 1:10. p220.2:pCOL2*a*(I)-CAT. 1 *m*g of p220.2 and 10 *m*g of pCOL2*a*(I)-CAT are added to 500 ul of 2XBBS (50 mM HEPES (Sigma, St. Louis, MO), 280 mM NaCl, 1.5 mM Na_2HPO4, pH 6.95) and 500 ul of 250 mM $CaCl_2$. The mixture is incubated at room temperature for 45 minutes to allow precipitates to form. The calcium phosphate-DNA precipitate is added dropwise to exponentially growing B16-F1 cells (1×10^6 cells/10 mm plate/10 ml growth media) in culture. The culture is then incubated at 37°C in 5% CO_2 for 15-24 hours. The medium is then removed and the cells trypsinized in 0.2% trypsin/0.02% EDTA (Gibco, Grand Island, NY), replated 1:10 and maintained in selection media containing 300 ug of hygromycin B/ml (Calbiochem., San Diego, CA). Hygromycin resistant clonal cell lines are picked by ring cloning, expanded in culture and examined for the incorporation of the transformation sensitive reporter.

E. Analysis of the clonal B16-F1 melanoma cell lines transfected with the pCOL2*a*(I)-CAT reporter unit

B16-F1 derived cell lines resistant to hygromycin are analyzed for the integration of the transfected COL2*a*(I)-CAT construct by Southern blot (Southern, J. Mol. Biol., 1975, 98: 503) and PCR analysis (Innis et al, PCR Protocols: A guide to methods and applications, Academic Press, 1990) of total genomic DNA prepared from the cells

(Sambrook et al., Molecular Cloning: A Laboratory Manual, CSHL, 1989). Since the transcriptional activity of the *a*-actin promoter is repressed by transformation, the CAT gene is not expressed in the B16-F1 derived cells.

A number of agents have been reported to induce differentiation of B16 melanoma cells, including dimethylsulfoxide (DMSO), butyric acid and dimethylthiourea (DMTU). Treatment of B16 melanoma cells with these agents results in the inhibition of growth, increase in melanin expression and alteration in cellular morphology consistent with a differentiated phenotype (Nordenberg et al., JNCI, 1985, 75: 891-895; Nordenberg et al., Exp. Cell. Res., 1986, 162: 77-85; Malik et al., Biol. Cell., 1987, 60: 33-40; Fax et al., Br. J. Cancer, 1991, 63: 489-494). To check that the CAT gene is activated under conditions which reverse the transformed stated, expression of CAT is monitored following treatment of the clonal cells in culture with DMSO (Sigma, St. Louis, MO) at 0.3%-2% (v/v) for 48 hours (cell viability is not affected by this treatment for up to 1.5% DMSO), butyric acid (Sigma, St. Louis, MO) at 0.2-2 mM for 48 hours and DMTU (Aldrich, Milwaukee, WI) at 10 mM for 48 hours. CAT expression is examined by Northern blot analysis of total RNA and by monitoring CAT enzyme activity according to the method of Gorman et al., MCB, 1982, 2: 1044-1051.

F. <u>In vitro anti-neoplastic drug screen with B16-F1 melanoma cells transfected with the COL2*a*(I)-CAT reporter</u>

Clonal B16-F1 derived cell lines capable of expressing the reporter (B16-F1(COL2*a*(I)-CAT)) are used. Exponentially growing B16-F1(COL2*a*(I)-CAT cells are trypsinized and plated into 96-well plates (Becton Dickinson,

anti-neoplastic drugs at graded concentrations are then added

to each well followed by 50 ul of complete media. The cells are incubated at 37°C. After different times of exposure to the test drugs, sample wells are aspirated, trypsinized and the cell extracts prepared for the assessment of CAT activity. Cell viability, cell numbers and morphology are also monitored. A tested drug restores expression of the CAT gene and is identified as a potential anti-neoplastic drug.

G. Transplantation of B16-F1 (COL2*a*(I)-CAT) melanoma cells into syngeneic hosts

Specific-pathogen free mice of the inbred strain C57BL/6 ($H\text{-}2^b$ haplotype), 8-12 week old males are obtained from The Jackson Laboratories (Bar Harbor, ME).

B16-F1(COL2*a*(I)-CAT) melanoma cell lines in exponential growth phase in culture are harvested by a 60 second treatment with 0.25% trypsin/0.02% EDTA solution (Gibco, Grand Island, NY). The cells are detached and 10% FBS (Gibco, Grand Island, NY) is added to arrest trypsin activity. For transplantation the B16-F1(COL2*a*(I)) cells are resuspended in Ca^{2+} and Mg^{2+} free Hanks balanced salts solution. The tumor cells may be transplanted by one of three routes: (i) subcutaneous: 50,000-100,000 viable cells are inoculated subcutaneously with a 26G needle; (ii) intraperitoneal: 100,000-500,000 viable cells are inoculated into the peritoneal cavity with a 26G needle; or (iii) intravenous: 25,000-100,000 viable cells in a volume of 0.2 ml are injected into the lateral tail vein. The recommended doses have been previously determined to produce a 100% tumor incidence for the parental melanoma cell line (Fidler et al., Eu. J. Cancer, 1973, 9: 223-227)

H. Assessment of tumorigenicity

B16 melanoma cells express melanin giving rise to black nodules. To test the tumorigenicity of the untreated B16-F1 derived melanoma cell lines (B16-F1(COL2*a*(I)), animals are sacrificed at various time points (up to 14 days) after

inoculation. Black nodules are counted (in the case of metastasis). Tumor growth is quantified by measurement of tumor dimensions and tumor volume as described (Klarlund & Forchhammer, PNAS USA, 1980, 77: 1501-1505).

I. In vivo of the activi of the transformation sensitive reporter unit in the transplanted tumor

The sensitivity of the transformation sensitive reporter unit in the transplanted tumor in vivo is examined by treating the animals with DMTU (25 ug/ml in phosphate buffered saline) administered intraperitoneally daily at a final dose of 250-1000 mg/kg body weight. The ability of a known anti-neoplastic drug, DMTU, to prevent or retard the development of the tumor is examined. Transplanted tumors are excised and the CAT activity in cell extracts prepared from the tumor before and during treatment of DMTU is assessed as previously described (Gorman et al., MCB, 1982, 2: 1044-1051). The activity of the transformation sensitive reporter unit is also examined by immunohistochemistry of tumor sections using CAT specific antibodies. The above treatment with DMTU results in restoration of expression of CAT activity, indicating that the reporter unit responds to the presence of an anti-neoplastic drug.

In a similar manner, the B16-F1(COL2α(I)-CAT) tumors transplanted into a syngeneic host (C57BL/6 mice) are used for testing the efficacy of potentially useful anti-neoplastic drugs. For example, the treatment with a test drug results in restoration of expression of CAT activity, thereby identifying the test drugs as an anti-neoplastic drug.

Example 6: Anti-Neoplastic Drug Screen Using Melanoma Cells Having COL2*a*(I) Promoter-CAT Reporter Unit And the E19 Coding Sequence Transplanted Into an Allogeneic or Xenogeneic Host

B16-F1 melanoma cells that have incorporated the transformation sensitive reporter unit COL2*a*(I) promoter-CAT into their genomes are transfected with an expression plasmid capable of expressing the adenovirus-2 E19 gene product, and the resulting cells are transplanted into an allogeneic or xenogeneic host for use in anti-neoplastic drug screening.

The procedures are as described in Example 5 except for the following.

A. Introduction of the Adenovirus-2 E19 gene into B16-F1(COL2*a*(I)-CAT) melanoma cells

The construction of an expression unit comprising the coding region of the adenovirus-2 E19 gene under the transcriptional control of the strong constitutive human CMV promoter in the vector pCDNA1NEO has been described in Example 4. This vector also contains a neomycin resistance gene that enables selection with G418 (Gibco, Grand Island, NY).

The DNA for the E19 expression unit is prepared as previously described, and transfected into exponentially growing B16-F1(COL2*a*(I)-CAT) melanoma cells in culture using the calcium phosphate precipitation method as previously described. Transfected cells are selected and maintained in complete media containing 500 ug/ml of G418.

B. Analysis of E19 expression and MHC class I presentation

Clonal cell lines derived from the B16-F1(COL2*a*(I)-CAT) melanoma line that have inherited the G418 resistance gene are examined for expression of Adeno-2 E19 by Northern blot

analysis of total RNA and Immunoblot analysis using E19 specific antisera (Wold et al., J. Biol. Chem., 1985, 260: 2424-2431; Persson et al., PNAS USA, 1980, 77: 6349-6353) on total protein prepared from the cells.

MHC class I expression and presentation on the cell surface of B16-F1(COL2*a*(I)-CAT) cells expressing the E19 protein are examined by conventional immunofluorescence microscopy using MHC class I specific H-2^b monoclonal antibodies (Pharmingen, San Diego, CA).

C. Transplantation of B16-F1(COL 2*a*(I)-CAT)E19 melanoma cells into allogeneic and xenogeneic animals

B16-F1(COL2*a*(I)-CAT)E19 melanoma cells are transplanted into Balb c mice (H-2^d haplotype) (The Jackson Laboratories, Bar Harbor, ME), C3H mice (H-2^k haplotype) (The Jacks . Laboratories, Bar Harbor, ME) and Fisher 344 rats (Charles River Laboratories, Wilmington, MA) as previously described. The growth and development of the untreated tumors are assessed as previously described for the transplanted tumors in C57BL/6 syngeneic hosts.

In a similar manner, the B16-F1(COL2*a*(I)-CAT)E19 tumors transplanted into allogeneic and xenogeneic hosts are used for testing the efficacy of potentially useful anti-neoplastic drugs. For example, the treatment with a test drug results in restoration of expression of CAT activity, thereby identifying the test drug as an anti-neoplastic drug.

Example 7: Anti-Neoplastic Drug Screen Using Human Breast Carcinoma Cells Having An *a*-Tropomyosin-3-Galactosidase Reporter Unit Transplanted Into An Immunodeficient or Host and An Immunocompromised Host

human breast carcinoma cells in culture and the resulting

cells are transplanted into an immunodeficient host and an immunocompromised host for use in anti-neoplastic drug screening.

A. MCF-7 human breast carcinoma cell line

MCF-7 is a human breast, infiltrating ductal carcinoma cell line originally isolated by Soule et al., J. Natl. Cancer Inst., 1973, 51: 1409-1416. The tumor cell line retains many of the characteristics of differentiated mammary epithelium including the expression of estrogen and progesterone receptors (Brooks et al., J. Biol. Chem., 1973, 248: 6251-6253) and *a*-lactobumin (Soule et al., J. Natl. Cancer Inst. 1973, 51: 1409-1416). MCF-7 has been used in vitro as a primary screen for anti-neoplastic agents (Finlay & Baguley, Eur, J. Cancer. Clin. Oncol., 1984, 20: 947-954) and is on the U.S. National Cancer Institute's recommended panel of human tumor cell lines for anticancer drug screening (Alley et al., Cancer Res. 1988, 48: 589-601; Scudiero et al., Cancer Res. 1988, 48: 4827-4833).

B. MCF-7 human breast carcinoma cell culture

The human breast carcinoma cell line MCF-7 is obtained from American Type Culture Collection (ATCC HTB22) (ATCC, Rockville, MD). The cells are maintained in Eagle's medium with non-essential amino acids, sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml of streptomycin, supplemented with 10% FBS (Gibco, Grand Island, NY). All cultures are maintained at 37°C, 5% CO_2 in a humidified chamber.

C. Transformation sensitive reporter unit construct

The preferred transformation sensitive reporter unit in this tumor cell line is an *a*-tropomyosin promoter-ß-galactosidase construct, the assembly of which has

been described in Example 1. The resulting construct is inserted into a plasmid vector carrying a neomycin resistance gene (e.g., pMClneo (Strategene, La Jolla, CA)). The *a*-tropomyosin promoter is repressed by transformation. The reversal of transformation by an anti-neoplastic drug presented to the cells leads to the restoration of transcriptional activity from the promoter resulting in ß-galactosidase expression.

D. Introduction of the transformation sensitive *a*-tropomyosin-ß-galactosidase reporter into MCF-7 cells in culture

DNA is introduced into MCF-7 cells by electroporation (Chu et al., 1987, 15: 1311-1325). Exponentially growing MCF-7 cells are harvested from culture and resuspended in 1xHeBS buffer (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCL, 0.7 mM Na_2HPO_4, 6 mM dextrose) at a concentration of 3 x 10^6 cells/ml. DNA comprising the *a*-tropomyosin-ß-galactosidase unit and the neomycin resistance gene are added to the cell suspension at a concentration of 20 ug/ml. The cells are then subjected to a single voltage pulse at 250 volts, 500 uF at room temperature. Upon returning to 4°C for 10 minutes, the cells are placed in culture in complete media for 48 hours. The transfected MCF-7 cells are then trypsinized and replated 1:10 in selection media containing 500 ug/ml G418 (Gibco, Grand Island, NY). G418 resistant clonal cell lines are picked by ring cloning and expanded in culture for analysis.

E. Analysis of the clonal MCF-7 derived cell line transfected with the *a*-tropomyosin-ß-galactosidase reporter unit

Southern blot and PCR analysis of total genomic DNA prepared from the cells.

The activity of the transfected reporter is examined by subjecting the MCF-7 (*a*-tropomyosin-ß-galactosidase) cells to treatments that have been previously reported to cause growth inhibition of the parental tumor (MCF-7) cell line in culture. The growth of MCF-7 in culture is readily inhibited by retinoic acid (Ueda et al., Cancer (Phila), 1980, 46: 2203-2209; Fontana et al., JNCI, 1987, 78: 1107-1112; Butler & Fontana, Cancer Res., 1992, 52: 6164-6167), anti-estrogens such as tamoxifen (Coezy et al., Cancer Res., 1982, 42: 317-323; Lykkesfeldt et al., Br. J. Cancer, 1984, 49: 717-722; Grenman, et al., J. Cancer Res. Clin. Oncol., 1991, 117: 223-226), suramin (Vignon et al., JNCI, 1992, 84: 38-42; Berthois et al., JNCI, 1992, 84: 1438-1439) and estrogen depletion (Sutherland et al., Cancer Res. 1983, 43: 3993-4006; Osborne et al., Cancer Res., 1984, 44: 1433-1439). In general, these treatments result in the growth arrest in the G0-G1 phase in the cell cycle (cytostatic) without producing cytotoxicity.

The expression of the ß-galactosidase reporter gene under the transcriptional control of the transformation sensitive *a*-tropomyosin promoter in the MCF-7 (*a*-tropomyosin-ß-galactosidase) tumor cells is examined upon treatment in culture with either tamoxifen (Sigma, St. Louis, MO) at 5 uM for 10 days, or Suramin (Bayer-Pharma., Puteaux, France) at 0.1 mM for 10 days. Tamoxifen is [Z]-2-[4-(1, 2-Diphenyl-1-butenyl)-phenoxy]-N, N-dimethylethanamine. It is an anti-estrogen that is used in the palliative treatment of breast cancer. (ICI, Wilmington, DE). Suramine (sodium) is 8, 8'-(Carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene) carbonylbis[imino]]-1,3,5-naphthenentri-sulfonic acid hexasodium salt. (Bayer/Miles, Pittsburgh, PA). In growth inhibition experiments resulting from estrogen depletion, MCF-7 (*a*-tropomyosin-ß-galactosidase) cells are cultured in steroid free media without phenol red. FBS is depleted of steroids by treatment with dextran-coated charcoal (Bonne &

Raynaud, Steroids, 1976, 27: 497-507) added to Eagle's minimal medium at a final concentration of 5%.

ß-galactosidase reporter gene expression is monitored by assaying enzyme activity in the presence of the chromogen X-Gal (Boehringer Mannheim, Indianapolis, IN) essentially as described (Bonnerot et al., PNAS USA, 1990, 87: 6331-6335).

F. In vitro anti-neoplastic drug screen with MCF-7 human breast carcinoma cells transfected with the *a*-tropomyosin-ß-galactosidase reporter

MCF-7 (*a*-tropomyosin-ß-galactosidase) tumor cells are plated at a concentration of 5×10^4 cells/well in each 35 mm wells of multiple six well plates in complete media. The cells are allowed to grow for 3-4 days before addition of test drugs. The medium is replenished daily to ensure that nutrient depletion does not affect cell growth. Fresh test drugs are added with each medium change. Cell growth and reporter gene expression are assayed as previously described. A tested drug reverses expression of the ß-galactosidase gene and is identified as a potential anti-neoplastic drug.

G. Transplantation of MCF-7 (*a*-tropomyosin-ß-galactosidase) human breast carcinoma cells into an immunodefficient host

5-6 week old, female, ovariectomized, athymic nude mice (nu/nu Balb/c) are obtained from Harlan Sprague-Dawley, Madison, WI. Mice are housed in a germ-free environment. All surgical manipulations are performed in a laminar flow hood. Surgical procedures are performed under Na-pentobarbitol anesthesia. Animals are implanted subcutaneously with a 0.25 cm Silastic capsule (0.078 inch

Louis, MO) as described (Kyprianou et al., Cancer Res., ...

51: 162-166). The implants have been previously demonstrated to deliver 200-400 pg/ml of 17B-estradiol for up to 3 months (Kyprianon et al., Cancer Res., 1991, 51: 162-166). Tumor cells are harvested from subconfluent monolayer cultures by 1 minute treatment with 0.25% trypsin and 0.02% EDTA. Cells are washed in supplemented medium and resuspended in Hank's balanced salt solution for inoculation. The E2-implanted ovariectomized animals are then injected subcutaneously with 2×10^6 viable MCF-7 (*a*-tropomyosin-ß-galactosidase) cells. Palpable tumors should develop within the first 2 weeks after inoculation. At graded intervals after transplantation of the tumor cells, animals are sacrificed and tumor growth and tumor pathology are characterized. The retention of mammary marker (estrogen and progestogen receptors) of the parental MCF-7 tumor are examined by immunoassay (ER-E1a & PR-E1A kits (Abbot Laboratories, Chicago, IL)) as described (Iino et al., Br. J. Cancer, 1991, 84: 1019-1024).

H. In vivo activity of the transformation sensitive reporter in the transplanted tumor

The sensitivity of the *a*-tropomyosin-ß-galactosidase reporter in the transplanted MCF-7 (*a*-tropomyosin-ß-galactosidase) tumor to growth inhibition, for example, to tamoxifen treatment is assessed. Tamoxifen (10 mg/ml in peanut oil) is administered intramuscularly twice weekly at doses of 5-50 mg/kg body weight (Mizukami et al., Anticancer Res., 1991, 11: 1333-1338). Tumor grafts are excised upon completion of treatment and ß-galactosidase expression is examined by staining fixed tumor sections in the presence of X-Gal (Bonnerot et al., PNAS USA, 1990, 87, 6331-6335).

In a similar manner, the MCF-7 (*a*-tropomyosin-ß-galactosidase) tumors transplanted into the immunodeficient host are used for testing the efficacy of potentially useful anti-neoplastic drugs. For example, the

treatment with a test drug results in reversal of expression of ß-galactosidase activity, thereby identifying the test drug as an anti-neoplastic drug.

I. Transplantation of MCF-7 (*a*-tropomyosin-ß-galactosidase) human breast carcinoma cells into an immunocompromised host

As an alternative, in vivo testing is performed by grafting the tumor cell line onto a xenogeneic host in which the immune system has been suppressed to prevent graft rejection essentially as described (Fingert et al., PNAS USA, 1984, 81: 7927-7931). Female Balb/c mice (Charles River Laboratories, Wilmington, MA) are immunosuppressed by treatment with cyclosporin A (Sandoz, East Hanover, NJ). Mice are injected subcutaneously with 80 mg/kg body weight in 0.1 ml of oil, each day starting 1-2 days after tumor transplantation. Tumor cell transplantation is carried out essentially as described above. The animals are ovariectomized and implanted with E2.

The MCF-7 (*a*-tropomyosin-ß-galactosidase) tumors transplanted into the immunocompromised host are used for testing the efficacy of potentially useful anti-neoplastic drugs. For example, the treatment with a test drug results in reversal of expression of ß-galactosidase activity, thereby identifying the test drugs as an anti-neoplastic drug.

It should be understood from the foregoing description that the prior art reveals the limitations of previous screens for the identification of anti-neoplastic drugs. While such screens have focused on identifying agents that kill rapidly growing cells, the current invention permits the in vivo identification of drugs that reverse neoplastic transformation without causing cell death. As a consequence,

Moreover, the combined in vitro/in vivo nature of the preferred embodiment of this invention permits an initial prescreen of putative anti-neoplastic drugs in a cell culture test system, with the concomitant advantages of ease, low cost, rapidity and large numbers, while also offering the advantages of analyzing the prescreened drugs in a live animal.

Unlike in vitro screens alone, this invention further provides access to obtaining information about drug availability in live animals. It can be determined if the drug reaches the tumor, the extent to which the drug penetrates the tumor, if the drug has different effects on different size tumors, if the drug has different effects on tumors at different stages of tumor progression, and if the drug's effects depend upon the state of vascularization of the tumor. For example, differential staining patterns within a tumor (e.g., whether the borders or centers are stained), resulting from a transformation sensitive reporter unit that produces a color change if the drug reverses neoplastic transformation, can provide useful information on drug delivery. Information about the tissue-specificity of a drug also can be obtained. For example, differential staining can indicate if a drug acts differently on tumors from different tissues.

The proper dosing of an anti-neoplastic drug also can be determined with this invention. In the preferred embodiment, the assay identifies a putative anti-neoplastic drug if the tumor changes color, even if the tumor might not have undergone a reduction in size. Once such a candidate drug is identified, the doses and/or timing of drug administration can be manipulated to achieve a desired result.

In addition, the invention allows an assessment of whether external conditions interfere with the efficacy of an anti-neoplastic drug. Unlike in vitro screens alone, this invention permits analysis of conditions specific to live animals, e.g., the animal's age, the animal's health, tissue

specificity, the degree of vascularization near the tumor, and synergistic effects with other drugs or nutrients ingested by the animal. Moreover, the invention can permit a determination of whether the efficacy of the drug depends upon *in vivo* modification of the drug.

Those skilled in the art will be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for testing the ability of a drug to interfere with development of neoplasia, comprising:

introducing a tumor cell into a recipient organism having an immune system, under a condition which reduces said recipient organism's rejection of said tumor cell, said tumor cell having a transformation sensitive reporter unit comprising a structural gene which expresses a product;

administering a drug to said recipient organism containing said tumor cell after said introducing; and

determining whether said drug has affected the expression of said structural gene that is part of said transformation sensitive reporter unit by assaying for said product of said structural gene.

2. The method of claim 1 wherein said tumor cell has an MHC gene which expresses MHC class I cell surface antigens and said condition comprises said tumor cell having the coding sequence for and expressing E19 protein so as to alter the presentation of MHC class I cell surface antigens on said tumor cell and allow introduction of said tumor cell into said recipient organism while reducing transplant rejection by said recipient organism's said immune system.

3. The method of claim 2 wherein said coding sequence for said E19 protein is carried on a vector which expresses said E19 protein.

4. The method of claim 2 wherein said condition further comprises said recipient organism being treated with an immunosuppressant drug.

5. The method of claim 2 wherein said condition further comprises said recipient organism being irradiated.

6. The method of claim 1 wherein said condition comprises said tumor cell having an inactive MHC gene so as to allow introduction of said tumor cell into said recipient organism while reducing transplant rejection by said recipient organism's said immune system.

7. The method of claim 6 wherein said condition further comprises said recipient organism being treated with an immunosuppressant drug.

8. The method of claim 6 wherein said condition further comprises said recipient organism being irradiated.

9. The method of claim 1 wherein said condition comprises said recipient organism being immunocompromised so as to allow introduction of said tumor cell into said recipient organism while reducing transplant rejection by said recipient organism's said immune system.

10. The method of claim 9 wherein said recipient organism being immunocompromised is obtained by irradiating said recipient organism.

11. The method of claim 9 wherein said recipient organism is immunocompromised by treatment of said recipient organism with an immunosuppressant drug.

12. The method of claim 11 wherein said immunosuppressant drug is selected from the group consisting of cyclosporin, azathioprine, FK-506, rapamycin, methotrexate and cyclophosphamide.

13. The method of claim 1 wherein said [illegible]

organism while reducing transplant rejection by said recipient organism's immune system.

14. The method of claim 1 wherein said condition comprises said recipient organism being syngeneic with said tumor cell so as to allow introduction of said tumor cell into said recipient organism while reducing transplant rejection by said recipient organism's said immune system.

15. The method of claim 1 wherein said transformation sensitive reporter unit includes a regulatory region operably linked to said structural gene, said regulatory region being repressed upon transformation of said recipient organism.

16. The method of claim 15 wherein said regulatory region is selected from the group of genes consisting of smooth muscle *a*-actin, skeletal muscle *a*-actin, myosin heavy chain, myosin light chain, *a*-tropomyosin, myo D, myogenin, muscle creatine kinase, collagen 2*a*(I), H-2K (class I MHC), neu and polyoma (early and late).

17. The method of claim 1 wherein said transformation sensitive reporter unit includes a regulatory region operably linked to said structural gene, said regulatory region being activated upon transformation of said recipient organism.

18. The method of claim 17 wherein said regulatory region is selected from the group of genes consisting of glucose transporter GLUTI and multidrug receptor MDR1.

19. The method of claim 1 wherein said structural gene is selected from the group of genes consisting of Escherichia coli LAC Z, Escherichia coli CAT and Firefly luciferase gene.

20. The method of claim 1 wherein said transformation sensitive reporter unit is the regulatory region of *a*-tropomyosin operably linked to the structural gene LAC Z.

21. The method of claim 1 wherein said transformation sensitive reporter unit is the regulatory region of collagen 2*a*(I) operably linked to the structural gene CAT.

22. The method of claim 1 wherein said transformation sensitive reporter unit is the regulatory region of smooth muscle *a*-actin operably linked to the structural gene LAC Z.

23. The method of claim 1, further comprising the initial steps of:

prescreening said drug by administering said drug to a culture of said tumor cells; and

determining whether said drug after said administering has affected the expression of said structural gene by assaying for said product of said structural gene.

24. A method for modifying an organism having an immune system for use in testing the ability of a drug to interfere with development of neoplasia, comprising:

selecting a tumor cell for transplantation, said tumor cell having a transformation sensitive reporter unit,

transplanting said tumor cell into said organism under a condition which reduces said organism's rejection of said tumor cell.

25. The method of claim 24 wherein said condition comprises said tumor cell having the coding sequence for and expressing E19 protein so as to alter the presentation of MHC class I cell surface antigens on said tumor cell and allow introduction of said tumor cell into said organism while reducing transplant rejection by said organism's said immune system.

immunosuppressant drug.

27. The method of claim 25 wherein said condition further comprises said organism being irradiated.

28. The method of claim 24 wherein said condition comprises said tumor cell having an inactive MHC gene so as to allow introduction of said tumor cell into said organism while reducing transplant rejection by said organism's said immune system.

29. The method of claim 28 wherein said condition further comprises said organism being treated with an immunosuppressant drug.

30. The method of claim 24 wherein said condition comprises said organism being immunocompromised so as to allow introduction of said tumor cell into said organism while reducing transplant rejection by said organism's said immune system.

31. The method of claim 24 wherein said condition comprises said organism being immunodeficient so as to allow introduction of said tumor cell into said organism while reducing transplant rejection by said organism's said immune system.

32. The method of claim 24 wherein said condition comprises said organism being syngeneic with said tumor cell so as to allow introduction of said tumor cell into said organism while reducing transplant rejection by said organism's said immune system.

33. A method of treating donor tumor cells having a transformation sensitive reporter unit to reduce recipient rejection caused by MHC class I cell surface antigens, said method comprising:

contacting said tumor cells with E19 protein to alter the presentation of MHC class I cell surface antigens on said tumor cells and allow introduction of said tumor cells into a recipient organism while reducing transplant rejection by said recipient organism's immune system; and

providing said tumor cells in a form for introduction into said recipient organism to reduce recipient rejection caused by MHC class I cell surface antigens.

34. The method of claim 33 wherein contacting said tumor cells with said E19 protein is carried out by intracellular production of said E19 protein by the presence in said tumor cells of DNA which carries and expresses the E19 coding sequence.

35. The method of claim 33 wherein said donor tumor cells are grown in cell culture and further comprising removing tumor cells after said contacting, from said donor tumor cell culture.

36. The method of claim 34 further comprising introducing said last mentioned cells into said recipient organism.

37. The method of claim 36 further comprising treating said recipient organism with an immunosuppressant drug.

38. The method claims 36 further comprising irradiating said recipient organism.

39. An organism having a transplanted tumor cell, said tumor cell having a transformation sensitive reporter unit.

41. The organism of claim 39 wherein said transplanted tumor cell has the coding sequence for and expresses E19 protein so as to alter the presentation of MHC class I cell surface antigens on said transplanted tumor cell.

42. The organism of claim 41 wherein the organism is a rodent.

43. The organism of claim 39 wherein said transplanted tumor cell has an inactive MHC gene.

44. A tumor cell for transplantation having a transformation sensitive reporter unit and the coding sequence for E19 protein.

45. A tumor cell for transplantation having a transformation sensitive reporter unit and an inactive MHC gene.

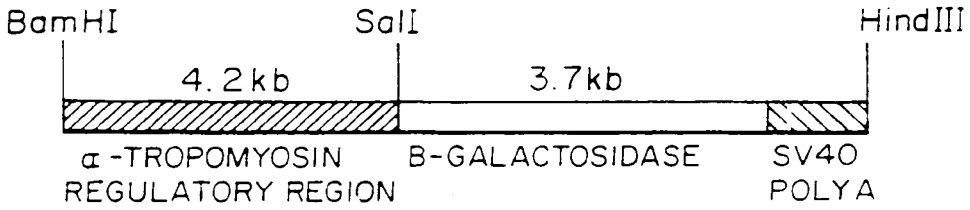

FIG. 1

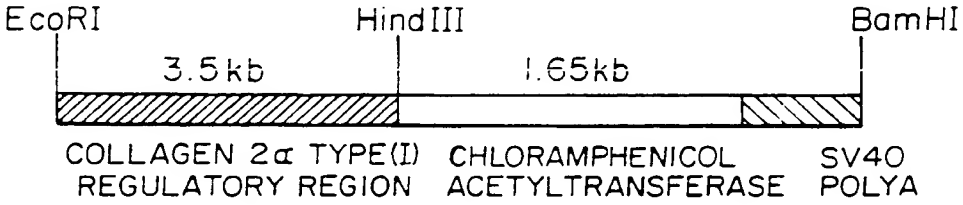

FIG. 2

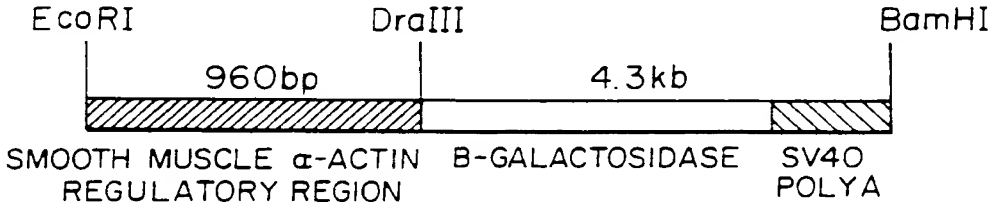

FIG. 3

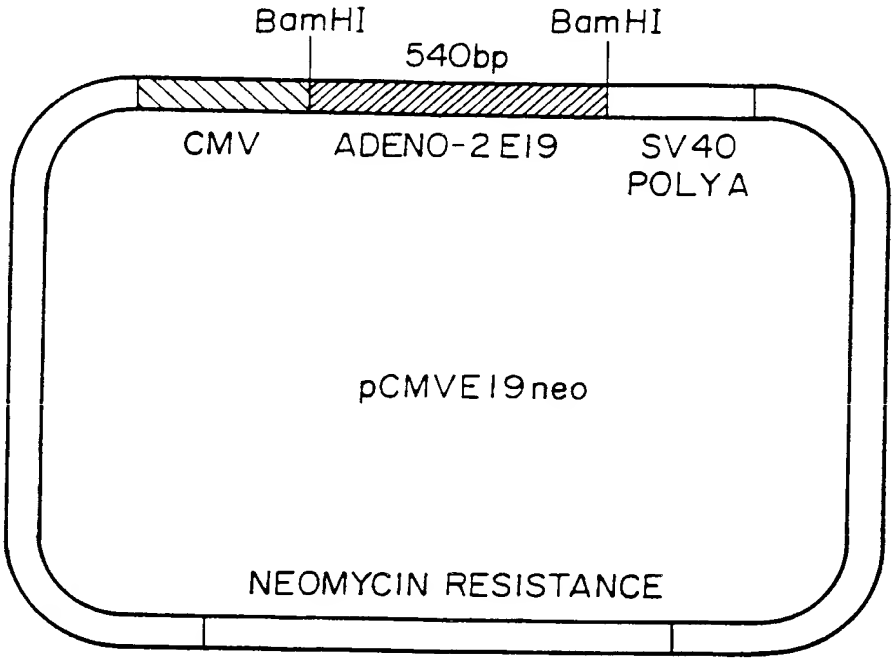
BamHI
BamHI
540bp
CMV
ADENO-2 E19
SV40
POLY A
pCMVE19neo
NEOMYCIN RESISTANCE

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/00237

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/34 A01K67/027 G01N33/50 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A01K C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PHARMACEUTICAL TECHNOLOGY vol. 15 , June 1991 pages 26 - 32 KUMAR, C.C. 'Setting up reporter-gene based assay systems for screening antineoplastic drugs' cited in the application see the whole document ---	1
P,Y	WO,A,93 23533 (TSI CORPORATION) 25 November 1993 cited in the application see the whole document --- -/--	1,6-22, 24, 28-32, 39,40, 43,45

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 06715 (SERAGEN INC.) 30 April 1992 see page 3, line 11 - page 9, line 22; claim 11	1,14-22, 24,28, 29,39, 40,43,45
Y	WO,A,91 01140 (CELL GENESYS) 7 February 1991 see the whole document	1,6, 15-22, 28,29, 32,45
P,Y	WO,A,93 02188 (OKLAHOMA MEDICAL RESEARCH FOUNDATION) 4 February 1993 see the whole document	1,6, 15-22,45
A	SCIENCE vol. 247, no. 4943 , 9 February 1990 , LANCASTER, PA US pages 715 - 718 COX, J.H. ET AL. 'Antigen presentation requires transport of MHC class I molecules from the endoplasmic reticulum' see the whole document	2
Y	JOURNAL OF CELLULAR BIOCHEMISTRY vol. SUPPL, no. 14A , 13 January 1990 , NEW YORK, USA page 345 DICK, J.E. ET AL. 'Engraftment of normal and leukemic human hematopoietic cells into immune-deficient SCID and BNX mice' see the abstract D 014	1,13, 15-22,31
Y	EP,A,0 517 199 (YEDA RESEARCH AND DEVELOPMENT COMPANY LTD) 9 December 1992 see the whole document	1,7-13, 15-22,30
Y	EP,A,0 322 240 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 28 June 1989 see the whole document	1,13, 15-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 94/00237

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9323533	25-11-93	AU-B-	4240293	13-12-93
WO-A-9206715	30-04-92	AU-A-	8947391	20-05-92
WO-A-9101140	07-02-91	AU-B-	633958	11-02-93
		AU-A-	6150590	22-02-91
		CA-A-	2037907	26-01-91
		EP-A-	0437576	24-07-91
		JP-T-	4501510	19-03-92
WO-A-9302188	04-02-93	CA-A-	2113089	04-02-93
		EP-A-	0591462	13-04-94
		AU-B-	4657993	24-01-94
		WO-A-	9400560	06-01-94
EP-A-0517199	09-12-92	NONE		
EP-A-0322240	28-06-89	AU-A-	2754888	29-06-89